Ratiometric Imaging of Extracellular pH in Bacterial Biofilms with C-SNARF-4

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pH in the extracellular matrix of bacterial biofilms is of central importance for microbial metabolism. Biofilms possess a complex three-dimensional architecture characterized by chemically different microenvironments in close proximity. For decades, pH measurements in biofilms have been limited to monitoring bulk pH with electrodes. Although pH microelectrodes with a better spatial resolution have been developed, they do not permit the monitoring of horizontal pH gradients in biofilms in real time. Quantitative fluorescence microscopy can overcome these problems, but none of the hitherto employed methods differentiated accurately between extracellular and intracellular microbial pH and visualized extracellular pH in all areas of the biofilms. Here, we developed a method to reliably monitor extracellular biofilm pH microscopically with the ratiometric pH-sensitive dye C-SNARF-4, choosing dental biofilms as an example. Fluorescent emissions of C-SNARF-4 can be used to calculate extracellular pH irrespective of the dye concentration. We showed that at pH values of <6, C-SNARF-4 stained 15 bacterial species frequently isolated from dental biofilm and visualized the entire bacterial biomass in in vivo-grown dental biofilms with unknown species composition. We then employed digital image analysis to remove the bacterial biomass from the microscopic images and accurately calculate extracellular pH values. As a proof of concept, we monitored the extracellular pH drop in in vivo-grown dental biofilms fermenting glucose. The combination of pH ratiometry with C-SNARF-4 and digital image analysis allows the accurate monitoring of extracellular pH in bacterial biofilms in three dimensions in real time and represents a significant improvement to previously employed methods of biofilm pH measurement.

Bacterial biofilms are involved in both beneficial and damaging processes. They are currently exploited in bioremediation, for example, in wastewater treatment plants (1), and in the production of biofuels or bioelectricity in microbial fuel cells (2, 3). Moreover, there is a multitude of symbiotic relationships between microbial biofilms and higher organisms that contribute to physiological homeostasis (4, 5). The functioning of the human gut, for example, depends strongly on microbial processes occurring in enteric biofilms (6, 7). On the other hand, biofilms are involved in food spoilage (8) and undesirable biofouling in the marine, industrial, and medical fields (9). They are the causative agents of many human diseases, such as wound and implant/device-related infections (10, 11), endocarditis (12), cystic fibrosis (13), periodontitis (14), and dental caries (15).

Bacterial biofilms possess a complex three-dimensional architecture (16). Reaction-diffusion interactions limit the penetration of solutes into and out of the biofilm (17), which leads to steep gradients of nutrients and metabolites and the creation of heterogeneous microenvironments that are colonized by organisms in different physiological states and with different biochemical requirements (18). Obligately anaerobic organisms, for example, can thrive in biofilms in overly aerobic environments, such as supragingival tooth surfaces or seawater (19, 20). Studying the architecture and bacterial metabolism in biofilms is of crucial importance to understanding, exploiting, and controlling biofilms.

The three-dimensional mapping of pH in bacterial biofilms merits intensive research. Biofilm pH has a central influence on the metabolic processes carried out in different areas of a biofilm. pH in microbial fuel cell biofilms, for example, has a significant impact on electric current production (21). In dental biofilms, pH at the biofilm-tooth interface is the key virulence factor for the development of caries lesions, one of the most prevalent diseases of mankind. For decades, pH in dental biofilms was recorded with pH-sensitive electrodes that only allowed bulk pH measurements (22–24). The development of pH microelectrodes with tip diameters as small as 10 μm (25) has improved the spatial resolution and made the recording of vertical pH gradients in dental biofilms possible (26, 27). pH microelectrodes also are employed to measure pH z profiles in wastewater treatment biofilms, microbial fuel cells, and in the context of biocorrosion (28–30). Still, the insertion of microelectrodes disturbs the biofilm mechanically, and it is not possible to measure horizontal pH gradients in biofilms.

In recent years, various fluorescence microscopic methods have been developed to overcome these problems and to map biofilm pH three dimensionally in real time. Two-photon excitation followed by time-gated fluorescence lifetime imaging (FLIM) uses pH-dependent differences in the fluorescence lifetime decay of, for example, carboxyfluorescein to determine local...
pH in a biofilm sample (31). pH-sensitive nanoparticles comprising both pH-sensitive and pH-insensitive dye molecules have been developed to quantify pH in biofilms by comparing the fluorescent emission of both dyes (32). Yet another approach is the use of ratiometric dyes, such as C-SNARF-4, that display different fluorescent emission spectra depending on the state of protonation of the dye. Calculating the fluorescent emission ratio at two different wavelengths allows the determination of local pH irrespective of dye concentration and compartmentalization (33, 34).

All fluorescence microscopic approaches have to face two major challenges. Although none of the methodologies rely on a homogeneous probe concentration for pH calculations, the fluorescent dye still has to penetrate the biofilm and bind in all extracellular areas with a sufficient concentration to allow for quantification. While small dye molecules readily diffuse into the biofilm, penetration proved to be a considerable problem for pH-sensitive nanoparticles. Hidalgo et al. reported that staining of the biofilm was not possible with nanoparticles of 70 nm and 30 nm in diameter, and 10-nm-sized particles mostly adhered to bacterial cells, which made it impossible to record pH in cell-free areas of the biofilm (32).

Additionally, it is crucial to differentiate between extracellular and intracellular pH in the biofilms. Intracellular pH will differ considerably from extracellular pH due to bacterial homeostasis, and in the case of dental caries, only extracellular pH will affect the underlying tooth. Both C-SNARF-4 and carboxyfluorescein penetrate bacterial cells at low pH. In previous studies by Vroon et al. (31), Hunter and Beveridge (34), and Franks et al. (35), no effort was undertaken to differentiate between the intra- and extracellular compartments; consequently, intracellular fluorescence contributed to the recorded pH values. Moreover, Hunter and Beveridge and Franks et al. employed genetically modified organisms expressing the fluorescent proteins green fluorescent protein (GFP) or mCherry to stain the biofilms. While the use of additional fluorescent stains to visualize bacteria in biofilms would permit us to differentiate between intra- and extracellular compartments, the application of fluorescent dyes other than the pH marker itself brings about the risk of fluorescent contamination and false measurements in the extracellular space.

In the present study, we therefore investigate the use of C-SNARF-4 in a dual function, as an intracellular bacterial stain and an extracellular pH marker. Since dental plaque is a classic example of an acid-producing biofilm, we investigated the staining abilities of C-SNARF-4 in the range of pH 4.0 to 8.0 on 15 different bacterial species frequently isolated from dental biofilm. Moreover, we used C-SNARF-4 to stain in vivo-grown dental biofilms of unknown composition and to monitor extracellular pH in these biofilms at the biofilm-substratum interface.

**MATERIALS AND METHODS**

**Bacterial strains.** Actinomyces naeslundii AK6, Actinomyces viscosus (CCUG 33710), Enterococcus fecalis (DSM 20478T), Lactobacillus paracasei subsp. paracasei (DSM 20020), Streptococcus gordoni (ATCC 10558T), Streptococcus mitis SK24, Streptococcus mutans (DSM 20523T), Streptococcus oralis (NCTC 7864T), and Streptococcus sanguinis (ATCC 10556T) were cultivated anaerobically on modified Columbia blood agar (36) and transferred to a liquid plaque medium (37) at 36.5°C until mid- to late exponential phase before experimental use.

Actinomyces naeslundii AK6 and Streptococcus mitis SK24 were kindly provided by M. Kilian, Department of Biomedicine, Aarhus University, Denmark.

**Visualization of bacteria in planktonic culture with C-SNARF-4.** Optical-bottom 96-well plates (Sigma-Aldrich, Brøndby, Denmark) were coated with a thin film of porcine gelatin (type A; 0.2% [wt/vol] in Milli-Q water; Sigma-Aldrich, Brøndby, Denmark) and dried overnight. Bacteria were washed twice in 0.9% sterile NaCl and adjusted to an optical density of 0.4 (550 nm). Bacterial suspensions were mixed with HEPES buffer solutions (50 mM, adjusted to pH 4.0 to 8.0 in steps of 0.5 pH units) at a ratio of 1:2 and set to settle in the 96-well plates for 1 h. All wells were washed twice with HEPES buffer of the right pH to remove nonadherent bacterial cells. Thereafter, bacteria were stained with C-SNARF-4 (Life Technologies, Nærum, Denmark) for 30 min and imaged with a confocal microscope (Zeiss LSM 510 META; Jena, Germany). The microscope was equipped with a 63× water immersion objective with a 1.2 numerical aperture (Plan Apochromat). A 543-nm laser line (250 to 300 μW) was used to excite C-SNARF-4, and fluorescence emission was monitored simultaneously within 576- to 608-nm (green) and 629- to 661-nm (red) intervals (META detector).

Initially, cells of S. mitis were incubated with different concentrations of C-SNARF-4 (20 μM, 30 μM, and 50 μM) at pH 4.0 to 8.0 to determine the ideal concentration for bacterial visualization. While 20 μM yielded sufficient contrast between cells and background, a concentration of 50 μM was judged to give the best cell/background ratio (see Fig. S1 in the supplemental material) and was used in all subsequent experiments. For all bacterial strains and pH values, images were acquired in three different microscopic fields of view, and the x-y positions were marked in the microscope software. All samples were counterstained with BacLight (Invitrogen, Taastrup, Denmark) according to the manufacturer’s instructions, and identical microscopic fields of view were imaged to check if all cells had been visualized by C-SNARF-4. BacLight was excited with 488-nm and 543-nm laser lines, and fluorescent emission was detected with the META detector set to 500 to 554 nm and 554 to 608 nm, respectively. Both channels in BacLight images were pseudocolored in red and both channels in C-SNARF-4 images in green, and the corresponding C-SNARF-4 and BacLight images were merged in Photoshop (Adobe, San Jose, CA). All experiments were repeated on another day.

**In situ biofilm growth.** Dental biofilms were grown in situ on custom-made nonfluorescent glass slabs (4 by 4 by 1 mm; Medimax, Braunschweig, Germany) with a surface roughness of 1.200 grit. Glass slabs were mounted slightly recessed on the buccal flanges of an individually designed lower jaw splint worn by a volunteer. The in situ model is described in more detail in Dige et al. (38). The splint was worn for periods of 48 h and was removed from the mouth only for oral hygiene and during intake of food or liquids other than water. The experimental protocol was approved by the Ethics Committee of Aarhus County (M-20100032).

**Visualization of bacteria in dental biofilms with C-SNARF-4.** After biofilm growth, the glass slabs were removed carefully from the splint. Biofilms (15 to 35 μm thick) were set to equilibrate for 30 min in custom-made wells filled with HEPES buffer (50 μM; PH 4.0 to 8.0 in steps of 0.5 pH units) and C-SNARF-4 (50 μM). For each biofilm, images were acquired directly above the biofilm-glass interface in three different microscopic fields of view, and the x-y positions were marked in the microscope software. Following counterstaining with BacLight, identical microscopic fields of view were imaged to check if the entire biofilm had been visualized by C-SNARF-4. Experiments were performed in duplicate. To test the visualization of thicker biofilms with C-SNARF-4, dental biofilm was sampled with sterile curettes, transferred to custom-made glass slabs, and incubated overnight in tryptic soy broth (Scharlau, Barcelona, Spain) at 35°C. Thereafter, biofilms were incubated for 45 min with sterile saliva (CCUG 24041T), and Veillonella parvula (CCUG 5123T) were cultivated anaerobically on modified Columbia blood agar (36) and transferred to a liquid plaque medium (37) at 36.5°C until mid- to late exponential phase before experimental use.

Actinomyces naeslundii AK6 and Streptococcus mitis SK24 were kindly provided by M. Kilian, Department of Biomedicine, Aarhus University, Denmark.
containing 0.4% (wt/vol) glucose and 50 μM C-SNARF-4. z-stacks spanning the entire height of the biofilms were acquired with an interslice distance of 3 μm. The experiments were performed in duplicate.

**Calibration of C-SNARF-4.** For confocal microscopic calibration, 100 μl of HEPES buffer solution (50 mM; adjusted to pH 4.5 to 8.5 in steps of 0.1 pH units), containing C-SNARF-4 at a concentration of 20 μM, were imaged in custom-made wells. For image acquisition, a Zeiss LSM 510 META (Jena, Germany) with a 40×/1.2-numeric-aperture water immersion objective (C-Apochromat) was used. The probe was excited with a 543-nm laser line (250 to 300 μW), and fluorescence emission was monitored simultaneously within 576- to 608-nm (green) and 629- to 661-nm (red) intervals (META detector), with the pinhole set to 2 Airy units (2-μm optical slice thickness). Images were 364 by 364 pixels (141 by 141 μm²) in size and were acquired with a pixel dwell time of 18 μs, line average of 2, 0.4 μm/pixel (zoom 1), and 12-bit intensity resolution. The procedure was repeated three times and was performed at 37°C with an XL incubator (PeCON, Erbach, Germany). A measurement was performed on unstained HEPES buffer for background subtraction and showed values similar to those for images of the stained buffer solution acquired with the 543-nm laser switched off. Therefore, the latter procedure was performed for every third pH value for background subtraction.

For ratio calculation, regions of 100 by 100 pixels were defined within each image, and the averages and standard deviations were determined using the LSM acquisition software. Subsequently, the ratio R, standard deviations, S_R, and standard errors of means, S_{pR}, were calculated for each pH value as previously described (39). The resulting values of R were plotted in SigmaPlot 10 (Systat Software, Inc., San Jose, CA, USA) and fitted to the following function:

\[
\text{pH} = \ln \left( \frac{1.61}{R - 0.0937} - 1 \right) \cdot 0.397 + 6.12 \tag{1}
\]

The calibration data and fitted curve are shown in Fig. S2 in the supplemental material.

**Biofilm pH imaging.** In situ-grown biofilms were placed in custom-made wells filled with C-SNARF-4 (50 μM) and salivary solution prepared according to the method of De Jong et al. (40). For biofilm pH imaging, the microscope was kept at 37°C with an XL incubator (PeCON, Erbach, Germany). Glucose was added to a concentration of 0.4% (wt/vol), and in each biofilm, one microscopic field of view was imaged every 30 s for 5 min immediately after the addition of glucose. Images were acquired at the bottom of the biofilm, just above the biofilm-glass interface, since pH in the bottommost layer of dental biofilms has the biggest influence on the caries process. For background subtraction, images were acquired with the 543-nm laser switched off. Thereafter, biofilms were counterstained with BacLight and the same fields of view were imaged to verify that the entire bacterial biomass was visualized with C-SNARF-4. Six replicate biofilms were studied.

**Biofilm pHe analysis.** In order to exclusively determine extracellular pH in the biofilms, the program daime (digital image analysis in microbial ecology, v.2.0) was employed to remove the entire bacterial biomass from all pictures (41). Red- and green-channel C-SNARF-4 biofilm images were exported separately as TIF files from the microscope software (LSM Image Examiner; Zeiss, Jena, Germany). In daime, green-channel C-SNARF-4 images were segmented with individually chosen brightness thresholds using the “Automatic segmentation” “Custom threshold” function. During segmentation, bacterial cells were recognized as objects. The object layer of the segmented images then was transferred to the corresponding red-channel C-SNARF-4 images. Thereafter, the object editor function was used to reject and delete all objects in both green- and red-channel images, leaving only the extracellular matrix in the images. In ImageJ (http://rsb.info.nih.gov/ij; v.1.47), background fluorescence was subtracted, the mean filter (radius, 1 pixel) was employed to compensate for detector noise, and the ratio between fluorescence intensities detected with the green (576 to 608 nm) and red (629 to 661 nm) filter sets was determined for every extracellular pixel. Average ratios and standard deviations were calculated for each field of view and converted to pH values according to equation 1.
RESULTS

Staining of bacterial species with C-SNARF-4. All 15 bacterial species included in the study were successfully stained with C-SNARF-4 at low pH (4.0 to 5.5). Confocal microscopic imaging showed that the dye was up-concentrated in the cells, which made them clearly distinguishable from the buffer background. Staining with 20 μM C-SNARF-4 yielded sufficient contrast between cells and the background, but a concentration of 50 μM was judged to give the best cell/background ratio (see Fig. S1 in the supplemental material). Counterstaining with BacLight and superimposition of the corresponding C-SNARF-4 and BacLight images showed that all cells took up C-SNARF-4 and that both viable and membrane-compromised cells were visualized. Figure 1 shows selected bacterial species stained with C-SNARF-4 and the corresponding BacLight images (see Fig. S3 in the supplemental material for the other investigated bacterial species).

At pH values above 6.0, the contrast between bacterial cells stained with C-SNARF-4 and the background decreased, and cells were more difficult to identify (Fig. 2 depicts examples). Table 1 lists the pH ranges suitable for the visualization of each organism.

Staining of dental biofilms with C-SNARF-4. In vivo-grown dental biofilms typically reached a thickness of 15 to 35 μm at the end of the 48-h growth phase. C-SNARF-4 was able to visualize bacteria in all layers of these dental biofilms kept at low pH (4.0 to 5.5). Counterstaining with BacLight proved that the entire bacterial biomass was visualized with C-SNARF-4 (Fig. 3A to D). As seen for planktonic cells, the contrast between cells and extracellular matrix was observed up to a depth of 75 μm (see Fig. S4 in the supplemental material). In deeper layers of the biofilm, the contrast decreased, and differentiation between extra- and intracellular compartments became difficult.

Calculation of extracellular pH in dental biofilms stained with C-SNARF-4. When C-SNARF-4-stained biofilms were incubated with 0.4% (wt/vol) glucose, the bacteria became visible within a very short time (<1 min). Digital image analysis with daine reliably identified and removed the bacterial biomass in all biofilm images, leaving only the extracellular compartment (see Fig. S5 in the supplemental material). Ratiometric pH analysis of the C-SNARF-4-stained biofilms incubated with glucose revealed that the bacterial biomass was visible already when the average pH in the microscopic field of view was well above pH 6. Figure 4 shows a biofilm with rapid acid production, where the average pH drops to 5.31 within 5 min (Fig. 4A to E), and a biofilm with moderate acid production, where average pH drops to 6.26 within 5 min (Fig. 4F to J). In both biofilms, the bacterial biomass is distinguishable from the first image (Fig. 4A and F), as shown by BacLight counterstaining (Fig. 4B and G). The average extracellular pHs at the time point the first images were taken were 6.49 (Fig. 4C) and 6.75 (Fig. 4H), respectively.

**TABLE 1** Visualization of bacterial species kept in HEPES buffer at different pH values with C-SNARF-4.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Visualization at pH ≤ 6.0</th>
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<tr>
<td></td>
<td>4.0</td>
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<tr>
<td><em>A. naeslundii</em> AK 6</td>
<td>+</td>
</tr>
<tr>
<td><em>A. viscosus</em> CCUG 33710</td>
<td>+</td>
</tr>
<tr>
<td><em>B. dentium</em> DSM 20436</td>
<td>+</td>
</tr>
<tr>
<td><em>E. faecalis</em> DSM 20478</td>
<td>+</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 10953</td>
<td>+</td>
</tr>
<tr>
<td><em>L. paracasei</em> DSM 20020</td>
<td>+</td>
</tr>
<tr>
<td><em>N. subflava</em> DSM 17610</td>
<td>+</td>
</tr>
<tr>
<td><em>P. gingivalis</em> ATCC 33277</td>
<td>+</td>
</tr>
<tr>
<td><em>P. intermedia</em> CCUG 24041</td>
<td>+</td>
</tr>
<tr>
<td><em>S. gordoni</em> ATCC 10558</td>
<td>+</td>
</tr>
<tr>
<td><em>S. mitis</em> SK24</td>
<td>+</td>
</tr>
<tr>
<td><em>S. mutans</em> DSM 20523</td>
<td>+</td>
</tr>
<tr>
<td><em>S. oralis</em> NCTC 7864</td>
<td>+</td>
</tr>
<tr>
<td><em>S. sanguinis</em> ATCC 10556</td>
<td>+</td>
</tr>
<tr>
<td><em>V. parvula</em> CCUG 5123</td>
<td>+</td>
</tr>
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</table>

* + indicates that a strain was visualized well at a given pH. (+) indicates that a strain was visualized but with low contrast compared to the extracellular background. - indicates that a strain could not be distinguished from the extracellular background at a given pH.
able to differentiate reliably between these compartments, but none of the hitherto published reports have addressed this problem sufficiently. Vroom et al. employed carboxyfluorescein for FLIM and claimed that the charged carboxy group prevents the dye from penetrating the cell (31). With a pKₐ of ~6.5, however, most of the molecule is protonated and, consequently, uncharged at low pH. In the published confocal microscopic images, bacterial cells are clearly visible in carboxyfluorescein-stained biofilms, which indicates that the dye was up-concentrated in the cells. As a consequence, the displayed pH values represent a mixture of pH in intra- and extracellular compartments. Likewise, Hunter and Beveridge, who were the first to use C-SNARF-4 in a bacterial biofilm, report that *Pseudomonas aeruginosa* cells internalized the dye and were discernible from the background (34). However, no measures were taken to remove intracellular areas from the microscopic images before pH calculation. Franks et al. employed C-SNARF-4 in current-generating *Geobacter sulfurreducens* biofilms (35), using the same approach as Hunter and Beveridge. Without differentiating between extracellular and intracellular compartments, pH was calculated for square areas in the analyzed microscopic fields of view, yielding an average of intra- and extracellular pH.

In order to truly map extracellular pH in a biofilm, it is critical to visualize the bacterial biomass in the biofilm and exclude it from pH calculations. Using a second, non-pH-sensitive dye to stain bacteria and later remove them via digital image analysis would induce the risk of fluorescent contamination of the extracellular space, which might compromise pH calculations. Hunter and Beveridge used green fluorescent protein (GFP) to visualize *P. aeruginosa*, and there is an overlap between the fluorescent emissions of C-SNARF-4 and GFP. The fluorescent emission of mCherry, used by Franks et al. to visualize *G. sulfurreducens* cells, peaks at 611 nm and shows considerable overlapping of the red spectrum emission of C-SNARF-4. Leakage of these dyes into the extracellular compartment compromises fluorescence intensity ratios calculated for pH measurement.

Therefore, in the present study, we investigated the use of C-SNARF-4 in a double function, as both a pH-sensitive ratiometric dye and a bacterial stain. We chose 15 different bacterial strains commonly isolated from supragingival dental biofilm and observed that C-SNARF-4 stained both viable and membrane-compromised cells at low pH (4.0 to 5.5) (Fig. 1; see also Fig. S3 in the supplemental material). Moreover, we showed that C-SNARF-4 stained the entire bacterial biomass in *in vivo*-grown dental biofilms of unknown bacterial composition (Fig. 3A to D and 4). This suggests that C-SNARF-4 serves as a universal bacterial stain.

Following image acquisition, we used the digital image analysis software daime to determine and remove the bacterial biomass from the microscopic images (see Fig. S5 in the supplemental material). Thereafter, fluorescent ratios could be calculated exclusively for the extracellular compartment and converted to pH values (Fig. 4). In principle, digital image analysis might well be employed to remove the extracellular space in the biofilm images and calculate fluorescent ratios inside bacterial cells. However, so far the calibration of C-SNARF-4 has been performed only for the extracellular space, using characteristic biofilm matrix components (34) and unstained biofilms as controls (39). Intracellular pH recordings would require a thorough calibration of the dye prior to quantitative interpretation of the fluorescence ratios (42).

**FIG 3** *In vivo*-grown dental biofilms, kept in HEPES buffer at pH 4.0 (A and B), pH 5.5 (C and D), and pH 7.0 (E and F), were stained with C-SNARF-4 (A, C, and E) and counterstained with BacLight (B, D, and F). In the range between pH 4.0 and pH 6.0, the entire bacterial biomass is reliably stained with C-SNARF-4 compared to the positive-control stain. Both viable and membrane-compromised cells are stained with C-SNARF-4. At pH values above 6.0, the contrast between C-SNARF-4-stained bacteria in the biofilms and the background decreases, and the bacterial biomass becomes hard to identify. Bars, 20 μm.
At higher pH (5.5 to 8.0), the staining properties of C-SNARF-4 decreased, and it became difficult to distinguish between bacterial cells and the extracellular space, both in pure cultures (Fig. 2) and in dental biofilms (Fig. 3E and F). C-SNARF-4 has a pKa of ~6.4, and only at low pH are the majority of the dye molecules protonated and uncharged. Only the uncharged molecule penetrates the bacterial membrane, binds to intracellular structures, and is up-concentrated in the bacteria. Therefore, ratiometric pH analysis with C-SNARF-4 is limited to biofilms in acidic environments.

Interestingly, cells in C-SNARF-4-stained dental biofilms incubated with glucose became visible immediately after exposure to 0.4% (wt/vol) glucose. Images in panels A and F were acquired right after the addition of glucose. In both cases, the bacterial biomass is clearly distinguishable, as verified by BacLight staining (B and G). Initial C-SNARF-4 images after removal of the bacterial biomass and ratiometric pH calculation. For visualization, false colors were assigned to each pH. Average pH in the images is 6.49 (C) and 6.75 (H). (D and I) The same fields of view as those shown in panels C and H 5 min after acquisition of the first images. (E and J) The bacterial biomass was removed, pH was calculated, and false colors were applied. (E) In the first biofilm, the average pH in the microscopic field of view dropped to 5.31 within 5 min. (J) In the second biofilm, acid production was lower and pH was 6.26 after 5 min. Bars, 20 μm.

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REFERENCES


